

The TCF4/ β -catenin pathway and chromatin structure cooperate to regulate *D*-glucuronyl *C*5-epimerase expression in breast cancer

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Keywords: D-glucuronyl C5-epimerase, GLCE, heparan sulphate proteoglycan, biosynthesis, tumor-suppressor gene, hypermethylation, chromatin structure, WNT signaling, TCF4/ β -catenin target, breast cancer

D-glucuronyl C5-epimerase (GLCE) is a potential tumor-suppressor gene involved in heparan sulfate biosynthesis. *GLCE* expression is significantly decreased in breast tumors; however, the underlying molecular mechanisms remain unclear. This study examined the possible epigenetic mechanisms for *GLCE* inactivation in breast cancer. Very little methylation of the *GLCE* promoter region was detected in breast tumors *in vivo* and in breast cancer cells (MCF7 and T47D) *in vitro* and *GLCE* expression in breast cancer cells was not altered by 5-deoxyazacytidine (5-aza-dC) treatment, suggesting that promoter methylation is not involved in regulating *GLCE* expression. Chromatin activation by Trichostatin A (TSA) or 5-aza-dC/TSA treatment increased *GLCE* expression by two to 3-fold due to an increased interaction between the *GLCE* promoter and the TCF4/ β -catenin transactivation complex, or H3K9ac and H3K4Me3 histone modifications. However, ectopic expression of TCF4/ β -catenin was not sufficient to activate *GLCE* expression in MCF7 cells, suggesting that chromatin structure plays a key role in *GLCE* regulation. Although TSA treatment significantly repressed canonical WNT signaling in MCF7 cells, it did not influence endogenous TCF4/ β -catenin mRNA levels and activated TCF4/ β -catenin-driven transcription from the *GLCE* promoter, indicating *GLCE* as a novel target for TCF4/ β -catenin complex in breast cancer cells. A correlation was observed between *GLCE*, *TCF4* and β -catenin expression in breast cancer cells and primary tumors, suggesting an important role for TCF4/ β -catenin in regulating *GLCE* expression both *in vitro* and *in vivo*. Taken together, the results indicate that *GLCE* expression in breast cancer is regulated by a combination of chromatin structure and TCF4/ β -catenin complex activity.

Introduction

Human D-glucuronyl C5-epimerase (GLCE, NM_015554) is one of the key enzymes involved in the biosynthesis of heparan sulfate proteoglycans (HSPGs), which play an integral role in cell-cell and cell-matrix interactions and signaling.¹⁻⁴ GLCE catalyzes the epimerization of D-glucuronic acid residues in the heparan sulfate (HS) polysaccharide chains into L-iduronic acid residues, which impart flexibility to the HS chains and facilitate their interaction with numerous extracellular ligands, including extracellular matrix (ECM) components and growth factors.⁵⁻⁷

GLCE activity is important for physiological processes such as B-cell maturation and differentiation,⁸ lymphangiogenesis,⁹ heparin biosynthesis by mast cells,¹⁰ development of zebra fish¹¹ and neuronal development.¹² Despite fragmentary knowledge regarding the biological functions of GLCE under normal physiological conditions, a crucial role of the gene is supported by data on the

neonatal lethality of Glce (-/-) knockout mice.^{9,13} These results suggest that functional disturbance of *GLCE* expression/activity and the consequent changes in composition and structure of cell surface HSPGs may be related to different pathological states, including cancer.¹⁴

We recently showed that GLCE is involved in malignant transformation and that *GLCE* expression is significantly decreased in breast tumors^{15,16} and small-cell lung cancer cell lines.¹⁷ It was also shown that the ectopic expression of *GLCE* inhibits cancer cell proliferation *in vitro* and tumor growth *in vivo*, suggesting a potential tumor-suppressor function for GLCE in breast and lung tissues.¹⁷⁻¹⁹ However, the molecular mechanism(s) underlying downregulation of GLCE in cancer remains unclear.

Previous studies show that the β -catenin-TCF4 transactivation pathway plays a major role in modulating *GLCE* expression in human colon carcinoma cell lines *in vitro*,²⁰ overexpression of the EJ-ras oncogene decreases D-glucuronosyl C-5 epimerase

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Submitted: 04/04/12; Revised: 06/18/12; Accepted: 06/20/12
<http://dx.doi.org/10.4161/epi.21199>

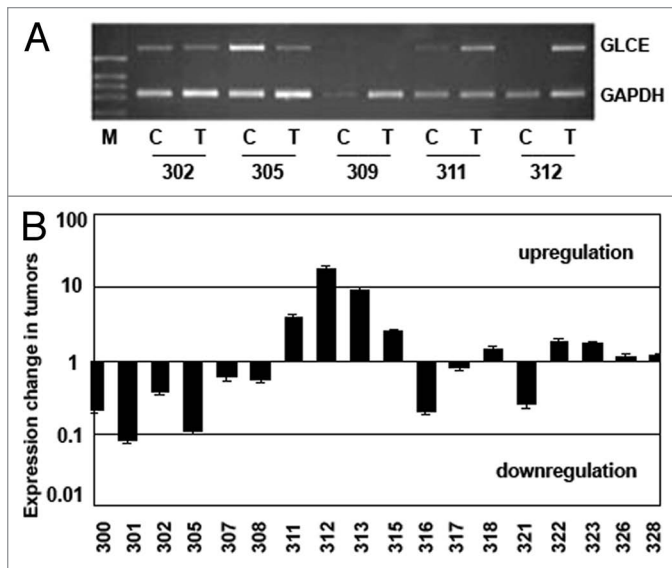


Figure 1. *GLCE* expression in the human breast tumors. (A) Representative RT-PCR electropherogram, (B) *GLCE* expression in tumor samples T, related to the match control breast tissue samples C, 1—similar expression in both samples, upper and down—upregulation or downregulation of *GLCE* expression, respectively, 301–328 patients.

mRNA levels in rabbit endothelial cells,²¹ and that miR-218 represses expression of *GLCE* in DLD colon cancer cells.²² To date, only these three papers have been published on this matter, and it is still unknown whether *GLCE* is regulated in a similar manner in other cancers, whether the mechanisms involved are applicable in vivo, and what other molecular mechanisms may be involved in the process.

Therefore, the present study examined the possible involvement of epigenetic mechanisms, as well as the β -catenin-TCF4 activating complex, in the downregulation of *GLCE* in breast cancer.

Results

The *GLCE* promoter is not methylated in human breast tumors. To check whether promoter hypermethylation could be responsible for changes in *GLCE* expression in breast tumors, 18 breast cancer clinical samples and appropriate control tissues with different *GLCE* expression levels were selected (18 matched pairs). *GLCE* expression level in the samples was determined by multiplex RT-PCR analysis with *GAPDH* gene as an internal control (Fig. 1).

Among the samples under investigation, there were both breast tumors with normal or elevated epimerase expression (compared with the matched control sample) and tumors with significantly decreased or lost *GLCE* expression.

The methylation status of *GLCE* promoter-associated CpG islands in the clinical samples and breast cancer cell lines (MCF7 and T47D) was analyzed using methylation-specific PCR (MSP) and bisulphite sequencing (Fig. 2).

Almost all clinical samples tested were completely unmethylated at the *GLCE* promoter region according to

methylation-specific PCR (Fig. 2B). Bisulphite sequencing of 16 samples (eight breast tumors and eight matched non-tumor control tissues) using two different primers pairs confirmed the MSP results (Fig. 2C). *GLCE* promoter methylation in the studied breast tumors was not more than 5–7% and did not show any specific methylation pattern in the different tumors. Also, the *GLCE* promoter was not methylated in MCF7 and T47D cells (Fig. 2D).

GLCE expression in breast cancer cells is activated by TSA but not by 5-aza-deoxycytidine treatment in vitro. To confirm the MSP and bisulphite sequencing data and to further study *GLCE* regulation, MCF7 and T47D cells were treated with the DNA demethylating agent 5-aza-deoxycytidine (5-aza-dC), the histone-deacetylase inhibitor Trichostatin A (TSA), or both. *GLCE* expression levels were determined by multiplex RT-PCR and qRT-PCR using *GAPDH* and β -actin, respectively, as a reference (Fig. 3).

The results showed that treatment with 5-aza-dC had no effect upon *GLCE* expression both in MCF7 and T47D cancer cells (data not shown), supporting the MSP and bisulphite sequencing results. Taken together, these data suggest that promoter methylation is not involved in the regulation of *GLCE* expression in human breast cancer cells in vitro or in breast tumors in vivo.

On the other hand, *GLCE* expression was clearly upregulated by 2–3-fold after TSA treatment, suggesting for the first time the possible involvement of chromatin structure in the regulation of *GLCE* expression in breast tumors.

Chromatin structure at the *GLCE* promoter is associated with activation of *GLCE* in breast cancer cells. A specific action of TSA on experimental breast cancer cells was confirmed by western blot analysis using antibodies to the active chromatin markers acetylated H3 lysine 9 (H3K9ac) and trimethylated H3 lysine 4 (H3K4me3), and the repressive chromatin marker, trimethylated H3 lysine 9 (H3K9me3). TSA treatment caused a marked increase in the histone isoforms H3K9ac and H3K4me3, which are associated with active chromatin, while the repressive histone marker H3K9me3 was not affected by the treatment (Fig. 4A). Next, chromatin immunoprecipitation (ChIP) analysis was performed to identify any direct involvement of TSA-activated chromatin in the upregulation of *GLCE* expression in TSA-treated MCF7 cells. Combined treatment with 5-aza-dC and TSA increased the precipitation of the *GLCE* promoter region by ChIP-grade antibodies to H3K9ac and H3K4me3, along with an increase in *GLCE* expression, suggesting the direct involvement of chromatin structure in *GLCE* regulation (Fig. 4B). Surprisingly, TSA treatment alone did not increase the association between the *GLCE* promoter and the H3K9ac and H3K4me3 histone modifications (whereas *GLCE* expression was increased in the cells). This suggests the involvement of other unidentified transcription factor(s), which may mediate increased *GLCE* expression in MCF7 cells upon TSA treatment.

Collectively, these results indicate that chromatin structures associated with histone modifications are involved in the regulation of *GLCE* expression in breast tissues, along with unknown TSA- or chromatin-activated positive *GLCE* regulators.

The Wnt/ β -catenin pathway in MCF7 cells is repressed by TSA. One of the primary candidates for the transcriptional regulation of *GLCE* expression in breast cancer cells may be the Wnt/ β -catenin pathway, which modulates *GLCE* expression in colon carcinoma cell lines in vitro.²⁰

To check the possible activation of Wnt signaling in MCF7 cells upon TSA treatment, we used the Wnt signaling pathway PCR array (SABiosciences), which identifies changes in the expression of 84 Wnt pathway-focused genes. The obtained data were analyzed using a companion scoring algorithm that converts gene expression data into quantitative measurements of pathway activity (Fig. 5).

The pathway activity score, based on gene expression changes, identified significant repression of WNT signaling activity in TSA-treated MCF7 cells (-0.73), mainly due to inactivation of the canonical WNT/Cttnn- β signaling pathway (upregulation of WNT inhibitors AXIN, WIF1, and downregulation of Frizzled co-receptor LRP5 and the WNT targets, MYC and CCND1).

Interestingly, the non-canonical WNT-Frizzled signaling pathway did not show any evident repression; to the contrary, increased expression of the characteristic proteins WNT1, WNT5 and WNT11 was detected along with the WNT antagonist, NKD1, which specifically blocks Cttnn- β but stimulates the JNK/WNT/PCP cascade. Taken together, the results suggest that TSA treatment of MCF7 cells causes an imbalance in the canonical and non-canonical WNT signaling pathways in favor of the WNT/PCP or WNT/ Ca²⁺ cascades.

Considering that previous data suggest that *GLCE* expression in human colon carcinoma cell lines is regulated via the β -catenin-TCF4 transactivation complex,²⁰ we analyzed the expression of the genes more carefully. According to the array, β -catenin (CTNNB1) expression was not changed in TSA-treated MCF7 cells (fold change, +1.08); however, TCF4 was not included in the array. Therefore, to further clarify the involvement of TCF4 and β -catenin in regulating *GLCE* expression in breast cancer cells, we studied the expression levels of these genes in breast cancer cells in vitro and in primary breast tumors in vivo.

***TCF4*, β -catenin, and *GLCE* expression correlate in breast tumors in vivo.** *TCF4*, β -catenin (CTNNB1) and *GLCE* expression was determined in primary breast tumors (matched tumor/control tissues) and MCF7 breast carcinoma cells both before

and after 5-aza-dC and/or TSA treatment by multiplex RT-PCR (Figs. 6 and 7).

In the breast cancer clinical samples (tumors and appropriate control tissues), a moderate positive correlation was found between *GLCE* and *TCF4* expression ($r = 0.62$, $p < 0.01$) and between *TCF4* and β -catenin expression ($r = 0.54$, $p < 0.01$) (Fig. 6A). This was even more evident when a tumor/control ratio (calculated for each matched pair) was used for correlation

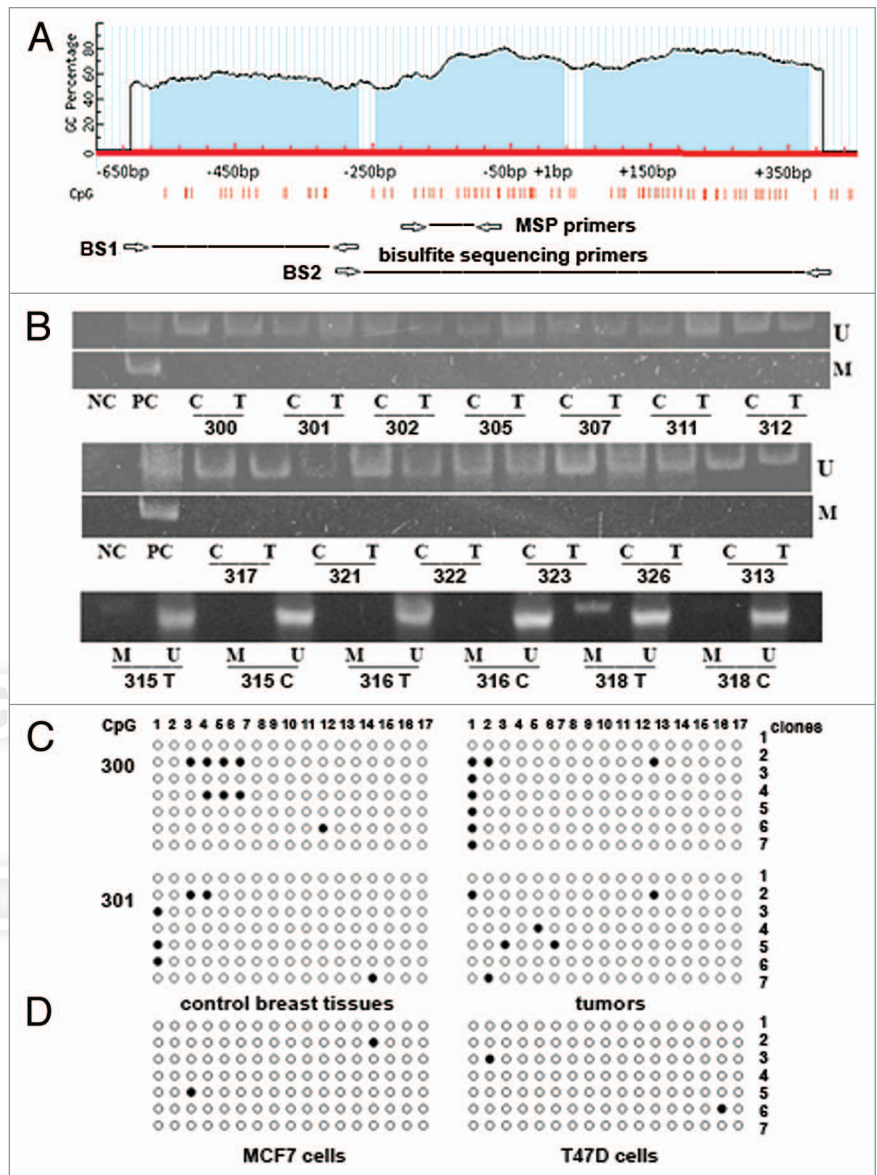


Figure 2. Methylation of *GLCE* promoter-associated CpG islands in human breast tumors and cancer cell lines. (A) Schematic showing the CpG islands in the *GLCE* promoter region. Location of methylation-specific and bisulphite sequencing PCR primers are indicated by arrows. (B) Methylation-specific PCR for the *GLCE* promoter region. 300–328, breast tumors, C and T, control and tumor breast tissues (match pairs from each patient); PC, positive PCR control; NC, negative PCR control; M, DNA marker; M and U, primers for methylated or unmethylated DNA sequences, respectively. (C and D) Bisulphite sequencing of breast tumors (C) and breast cancer cell lines MCF7 and T47D (D) using the BS1 primer pair. 300 and 301 patients, seven different *E. coli* clones (1–7) were sequenced for each breast tumor or cell line, open and black circles are non-methylated and methylated CpG dinucleotides, respectively.

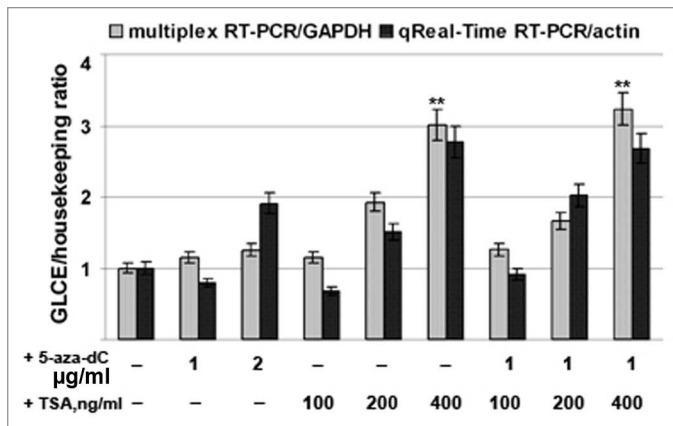


Figure 3. Activation of *GLCE* expression in MCF7 breast carcinoma cells by 5-aza-deoxycytidine or Trichostatin A. The intensity of the amplified *GLCE* DNA fragments was normalized to that of *GAPDH* (multiplex RT-PCR) and β -*actin* (Taqman-based qReal-Time RT-PCR). Bars represent the mean \pm SD from triplicate experiments, ** $p < 0.01$, p values between MCF7-TSA400-treated MCF7 and MCF7-aza-TSA400-treated MCF7 data points (OriginPro 8.1). 5-aza-dC, 5-aza-deoxycytidine; TSA, Trichostatin A.

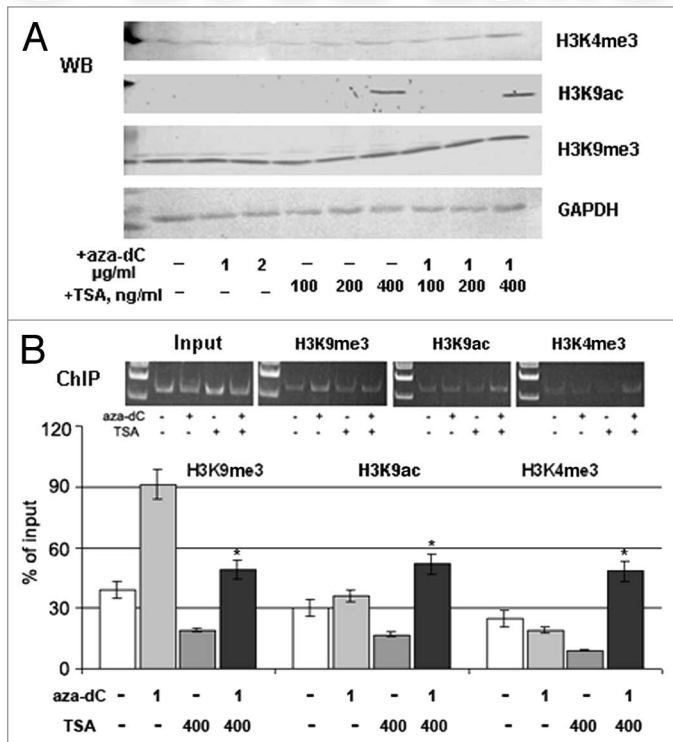


Figure 4. Involvement of chromatin structure in *GLCE* activation upon TSA treatment. (A) Changes in the expression of different histone modifications in MCF7 cells after 5-aza-dC and TSA treatment. Western blot (WB) analysis with specific antibodies. (B) Chromatin immunoprecipitation assay for the *GLCE* promoter region. Chromatin DNA was immunoprecipitated with ChIP-grade antibodies and DNA fragments corresponding to 183 bp in the *GLCE* promoter region were amplified by PCR. The amount of immunoprecipitated DNA was normalized to that of the input DNA. * $p < 0.05$, p values between MCF7-aza-TSA400-treated MCF7 data points.

analysis (Pearson correlation coefficients $GLCE/TCF4 = 0.86$, $p < 0.05$; $GLCE/\beta$ -*catenin* = 0.68, $p < 0.05$; and $TCF4/\beta$ -*catenin* = 0.81, $p < 0.01$) (Fig. 6B).

The data clearly showed a positive correlation between *GLCE*, *TCF4* and β -*catenin* expression changes in breast tumor tissues, supporting the involvement of the *TCF4*/ β -*catenin* complex in *GLCE* regulation in breast cancer in vivo.

TSA treatment induces the interaction between the *TCF4*/ β -*catenin* complex and the *GLCE* promoter in MCF7 cells in vitro. The multiplex RT-PCR data for *TCF4* and β -*catenin* expression in MCF7 cells confirmed the WNT array results and showed constitutive β -*catenin* and *TCF4* expression in these cells (Fig. 7A and B).

5-aza-dC or TSA treatments did not affected β -*catenin* expression significantly whereas *TCF4* expression was down-regulated in 5-aza-dC- or TSA-treated MCF7 cells. Combined treatment with 5-aza-dC and TSA increased *TCF4* expression by up to 2-fold and *GLCE* expression by up to 1.7-fold. A moderate positive correlation between *GLCE*, *TCF4* and β -*catenin* changes expression was observed in treated breast carcinoma cells in vitro (Pearson correlation coefficients $GLCE/TCF4 = 0.61$, $p < 0.07$; $GLCE/\beta$ -*catenin* = 0.71, $p < 0.05$; and $TCF4/\beta$ -*catenin* = 0.50, $p < 0.1$), which supported the in vivo results (Fig. 6).

To confirm the direct involvement of the *TCF4*/ β -*catenin* transactivation complex in *GLCE* regulation in breast cancer cells, chromatin immunoprecipitation was performed with anti-*TCF4* or anti- β -*catenin* antibodies on control (low *GLCE* expression) and TSA-treated (increased *GLCE* expression) MCF7 cells. Although TSA treatment did not influence β -*catenin* mRNA level and even decreased *TCF4* mRNA (Fig. 7A and B), the interaction between *TCF4* and β -*catenin* and the *GLCE* promoter sequence was significantly increased at the *TCF4*-responsive region (Fig. 7C). Specificity of the interaction was confirmed using primer pairs for *TCF4*-non-responsive promoter regions (primers P2 and P3).

All together, the results indicate an important role for chromatin structure in the formation of active *TCF4*/ β -*catenin* transcriptional complex at *GLCE* promoter.

Upregulation of *TCF4* and β -*catenin* expression alone is not sufficient to activate *GLCE* expression in MCF7 cells in vitro. To study the effects of *TCF4*/ β -*catenin* activation on *GLCE* expression in breast cancer in more detail, MCF7 breast carcinoma cells were transfected with *TCF4*- and/or β -*catenin*-expressing plasmids followed by *GLCE* expression analysis. Immunofluorescent staining showed that *TCF4* and β -*catenin* expression was significantly increased in the transfected cells with high co-transfection efficiency. Surprisingly, ectopic overexpression of *TCF4* and β -*catenin* did not increase *GLCE* expression in transfected MCF7 cells, suggesting that the *TCF4*/ β -*catenin* complex alone was not sufficient to activate *GLCE* in breast cancer cells (data not shown). However, chromatin activation by TSA (Fig. 4) was sufficient to recruit the *TCF4*/ β -*catenin* transactivation complex to the *GLCE* promoter region (Fig. 7C) and increase expression of the gene (Fig. 7A and B). Taken together, these results indicate that chromatin structures associated with H3K9ac and H3K4me3 histone modifications, together with the

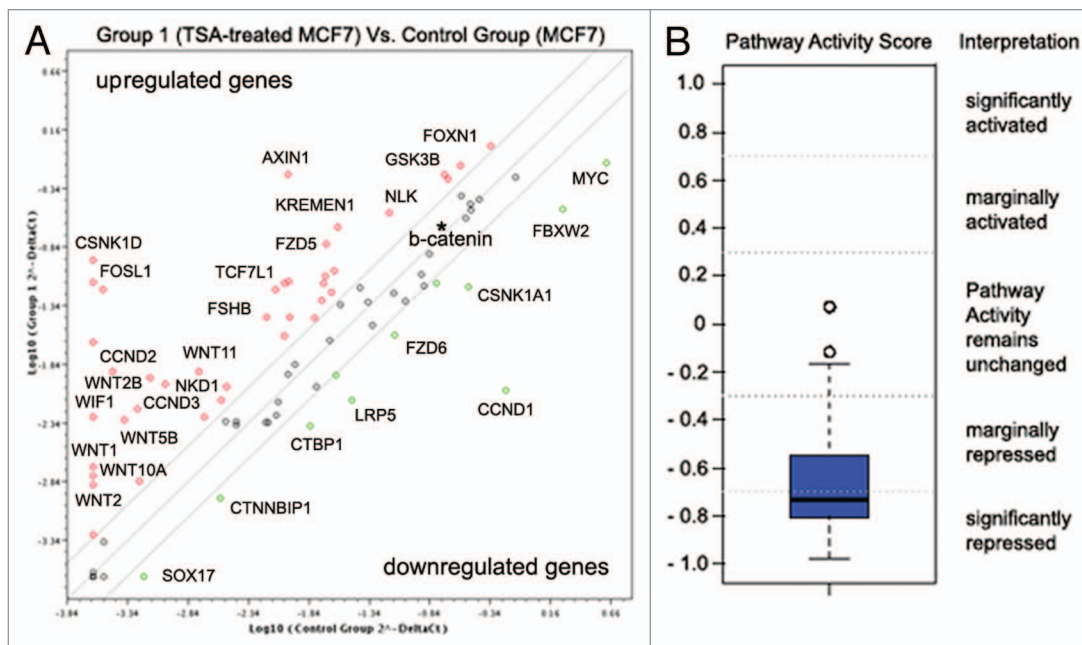


Figure 5. Wnt pathway repression in TSA-treated MCF7 breast cancer cells. (A) Fold changes in the expression of 84 genes relevant to the Wnt signaling pathway. Fold change in normalized gene expression in the Test Sample (TSA-treated MCF7 cells) divided by the normalized gene expression in the Control Sample (MCF7 cells). The middle line shows similar expression in both groups with 2-fold change boundaries. (B) Pathway activity score for Wnt signaling in experimental samples relative to control samples.

TCF4/ β -catenin complex, contribute to the regulation of *GLCE* expression in human breast cancer tissues.

Discussion

Epigenetic regulation is one of the fundamental ways of organizing and coordinating the activity of numerous genes and cellular pathways.^{23,24} Since inactivation of tumor-suppressor genes often occurs as a consequence of hypermethylation within the promoter regions,²⁵ and *GLCE* is a potential tumor-suppressor gene,^{17,18} hypermethylation of the *GLCE* promoter was our primary candidate for *GLCE* inactivation in breast cancer. The hypothesis was supported by the fact that hypermethylation is also involved in regulating the expression of a number of HSPGs and HS biosynthetic enzymes, such as the tumor-suppressor gene glycosyltransferase *EXT1*,²⁶ the 3-OST sulfotransferase genes (*3-OST1*, *3-OST2* and *3-OST3A*) in H-EMC-SS chondrosarcoma cells²⁷ and *3-OST-2* in human breast cancer cell lines and in breast, colon, lung and pancreatic cancers.²⁸ Promoter hypermethylation was identified as one of the mechanisms underlying HS sulfatase-1 (*HSulf-1*) gene silencing in human breast and gastric cancers²⁹ and HSPG glypican-3 (*GPC3*) silencing in human breast cancer.³⁰ Surprisingly, no methylation of the *GLCE* promoter was found in breast tumors or breast cancer cell lines, identifying *GLCE* as the first gene belonging to the HSPG biosynthetic machinery that is not regulated by promoter methylation.

According to the results of the present study, a complex combination of chromatin structure, TCF4 expression and, possibly, other TSA- or chromatin-activated positive *GLCE* regulators, controls the transcriptional regulation of *GLCE* expression in breast

cancer. Of these, chromatin remodeling appears to be the key factor that keeps expression under control because only the TSA-activated TCF4/ β -catenin complex (but not ectopic TCF4/ β -catenin expression) upregulated *GLCE* expression in breast cancer cells. This is in line with published data regarding the involvement of chromatin structure/histone modifications in regulating the expression of some glycosyltransferases and tumor-suppressor genes. It was shown that N-acetylglucosaminyltransferase IX and several other neural glycosyltransferase genes are regulated by epigenetic histone modifications.³¹ Promoter methylation-associated gene silencing of the *TMS1* tumor-suppressor gene is accompanied by H3K9 hypoacetylation and trimethylation, which are reversed after 5-aza-dC and/or TSA treatment. Interestingly, TSA is more efficient than 5-aza-dC in inducing *TMS1* expression, and the combination of 5-aza-dC and TSA results in marked synergistic reactivation of the gene.³² Similar data were shown for the *RIZ1* tumor-suppressor gene; neither 5-aza-dC nor TSA reversed promoter methylation, but contributed to the dynamic conversion of trimethylated to acetylated H3K9 at the promoter, and restored *RIZ1* expression with a marked synergistic effect.³³ A complex interplay between promoter hypermethylation and chromatin activation is supported further by data showing that, despite CpG dinucleotide hypermethylation in *TCF4* exon 1 (strongly associated with gene silencing), treatment with TSA restored *TCF4* expression in TCF4-silenced gastric cancer cell lines.³⁴

Chromatin regulation was shown for the tumor-suppressor gene *DKKI* (a potent inhibitor of the Wnt signal transduction pathway), which did not show significant levels of methylation in the promoter region; however, its expression was increased

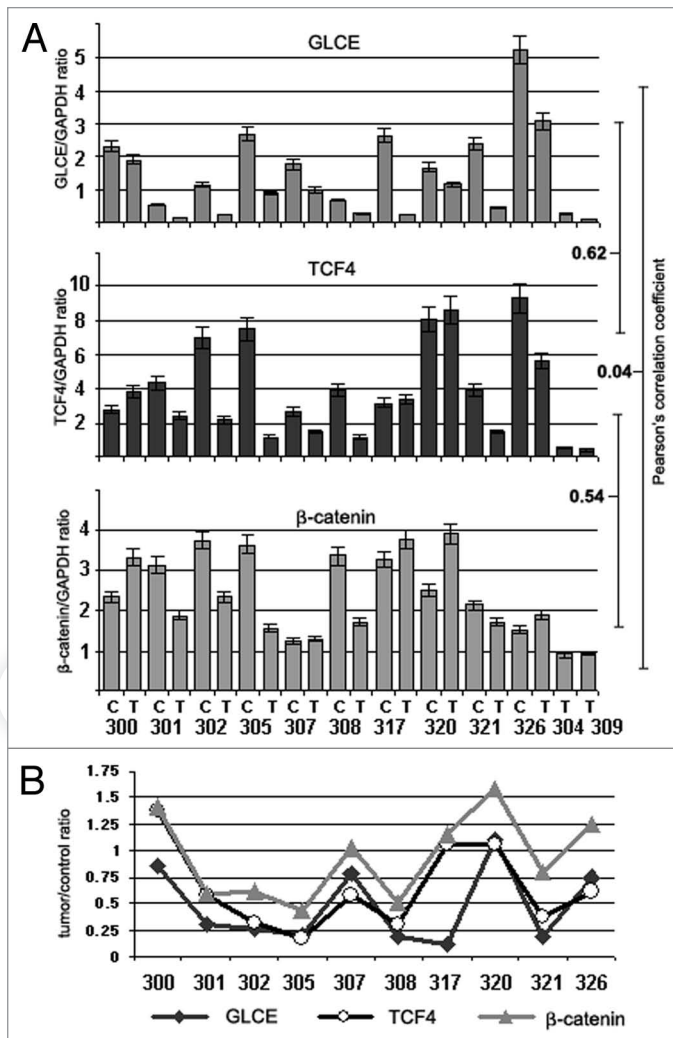


Figure 6. *GLCE*, *TCF4* and β -*catenin* expression in human breast tumors in vivo. (A) The intensity of the amplified DNA fragments was normalized to that of *GAPDH* (multiplex RT-PCR). Bars represent the mean \pm SD from triplicate experiments; Pearson's correlation coefficients are shown (OriginPro 8.1). (B) A tumor/control ratio for *GLCE*, *TCF4* and β -*catenin* expression calculated for each clinical sample. 300–326, breast tumors; C and T, control and tumor breast tissues (match pairs from each patient).

significantly upon TSA treatment due to increased histone acetylation and chromatin accessibility.³⁵

Collectively, these data strongly suggest an involvement for chromatin structure in regulation of the expression of tumor-suppressor genes along with promoter hypermethylation.

A pivotal role for chromatin remodeling in *GLCE* activation in breast cancer cells is supported by our results regarding TSA-induced *TCF4*/ β -*catenin* activation (Fig. 7C) or ectopic *TCF4*/ β -*catenin* expression in breast cancer cells. Specific over-expression of the *TCF4*/ β -*catenin* complex (transient transfection with the appropriate plasmids) was not sufficient to increase *GLCE* expression in MCF7 cells, whereas chromatin activation by TSA facilitated the interaction between endogenous *TCF4*/ β -*catenin* complex and the *GLCE* promoter, which successfully induced *GLCE* expression. The synergistic action of 5-aza-dC/

TSA suggests also the possible involvement of some other transcriptional co-factor(s), which is indirectly activated by 5-aza-dC/TSA or chromatin remodeling.³⁶

Another important aspect to be discussed is the functional role of the *TCF4*/ β -*catenin* complex and WNT signaling in modulating *GLCE* expression in cancer cells. A major role for the β -*catenin*-*TCF4* transactivation pathway in modulating *GLCE* expression was identified in human colon cancer cells.²⁰ Interestingly, *GLCE* expression in these cells was highly correlated with the degree of β -*catenin*-transcription complex activation; co-transfection with excess *TCF4* and β -*catenin* activated the *GLCE* promoter in the reporter assay and increased *GLCE* expression in HCT116 cells constitutively expressing low levels of β -*catenin* and *TCF4*. By contrast, neither β -*catenin* nor β -*catenin*/*TCF4* were able to upregulate *GLCE* expression in SW480 cells, which constitutively express higher levels of the β -*catenin*/*TCF4* complex and *GLCE*, although *TCF4* alone did increase *GLCE* promoter activity in a reporter assay.²⁰ In this study, *TCF4*/ β -*catenin*-expressing MCF7 breast cancer cells were used, and no *GLCE* upregulation was observed upon co-transfection of β -*catenin* and *TCF4*. Dependence of the *TCF4* effect on target gene expression from the overall WNT pathway activity in experimental cells was also shown for osteopontin expression in MCF10AT and Rama37 breast cancer cells, which possess differential Wnt signaling competency.³⁷ Taken together, the results of the present study underline the importance of endogenous *TCF4*/ β -*catenin* expression levels in the *TCF4*/ β -*catenin*-related experiments, and identify *GLCE* as a novel downstream target of WNT/ β -*catenin* signaling in breast cancer cells.³⁸

On the other hand, a feedback loop may exist between *GLCE* expression and Wnt signaling through *GLCE* substrates, such as HSPGs. It was shown that HSPGs are involved in the activation of specific Wnt pathways:³⁹ the HSPG glypican-4 (*GPC4*) regulates β -*catenin*-dependent and -independent Wnt signaling by concentrating Wnt3a and Wnt5a close to their specific receptors within different membrane microdomains⁴⁰ and the HSPG biosynthetic enzyme, 2-*OST*, is an essential component of the canonical Wnt signaling pathway during zebrafish development.⁴¹ Our observations that ectopic *GLCE* expression in stably-transfected MCF7 cell clones decreases β -*catenin*, but not *TCF4*, expression in cells suggests the possible involvement of *GLCE* in the regulation of WNT signaling in breast cancer cells (unpublished data).

Interestingly, TSA treatment significantly repressed WNT signaling activity in MCF7 cells (mainly due to selective inactivation of the canonical WNT/Ctnn- β signaling pathway) and activated the expression of its downstream target, *GLCE*, due to chromatin remodeling. Possibly, simultaneous repression of canonical WNT signaling pathway and upregulation of the tumor-suppressor gene, *GLCE*, in breast cancer cells may be one of the potential molecular mechanisms underlying the anti-tumor effects of TSA.⁴²⁻⁴⁴

Taken together, the presented data show complex transcriptional regulation of *GLCE* expression in breast cancer cells, in which the combined effects of chromatin structure and

TCF4/ β -catenin complex activity are necessary to maintain *GLCE* expression. Possibly, low *GLCE* expression in breast cancer cells is due to the fact that the *GLCE* promoter is inaccessible to the TCF4/ β -catenin transactivation complex because of a change in the chromatin structure in cancer cells.

Materials and Methods

Patients and tissue samples. All tissue samples were obtained from primary breast tumors during radical surgery at the Central Municipal Hospital N1, Novosibirsk, Russia. Tissues were “snap-frozen” in liquid nitrogen and stored at -70°C . Regions were manually dissected from the frozen blocks to provide a consistent tumor cell content of more than 70% for analysis. The prevalent histological type of the tumors was duct infiltrating cancer, with different degrees of malignancy. Most patients were at the second stage of malignancy progression according to the TNM formula. All patients provided written informed consent and the study protocol was approved by the Local Ethics Committee in accordance with the Helsinki Declaration of 1975.

Cell lines, cell culture and 5-aza-dC/TSA treatment. The human breast cancer cell lines, MCF7 and T47D, were obtained from the Karolinska Institute (Stockholm, Sweden). Cells were maintained in IMDM medium supplemented with 2 mM L-glutamine, 100 units penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO_2 . For analysis, cells were harvested using trypsin/EDTA (Sigma). Treatment with deoxyazacytidine (5-aza-dC, 1 or 2 $\mu\text{g}/\text{ml}$) or Trichostatin A (TSA, 100, 200 or 400 ng/ml) was performed by incubating the cells with the drugs for 72 h or 24 h, respectively. For combined treatment, the cells were incubated with 5-aza-dC (1 $\mu\text{g}/\text{ml}$) for 48 h followed by TSA (100, 200 or 400 ng/ml) for an additional 24 h. Cells were harvested using trypsin/EDTA.

Analysis of *GLCE*, *CTNNB1* and *TCF4* expression using multiplex RT-PCR. Multiplex RT-PCR analysis of *GLCE* expression was performed as described previously.^{17,18} Briefly, total RNA was extracted from the cells using the PureLink Total RNA Purification System (Invitrogen), cDNA was synthesized from 1–2 μg of total RNA using a First Strand cDNA Synthesis kit (Fermentas), and 1/10th of the product was subjected to PCR analysis. The following conditions were used for multiplex RT-PCR for *GLCE*: 95°C for 10 min, 95°C for 15 sec, 59°C for 15 sec, and 72°C for 1 min, with a final elongation step at 72°C for 10 min using a Tercik PCR machine (DNA-technology, Russia). The total reaction volume was 20 μl . *GLCE*

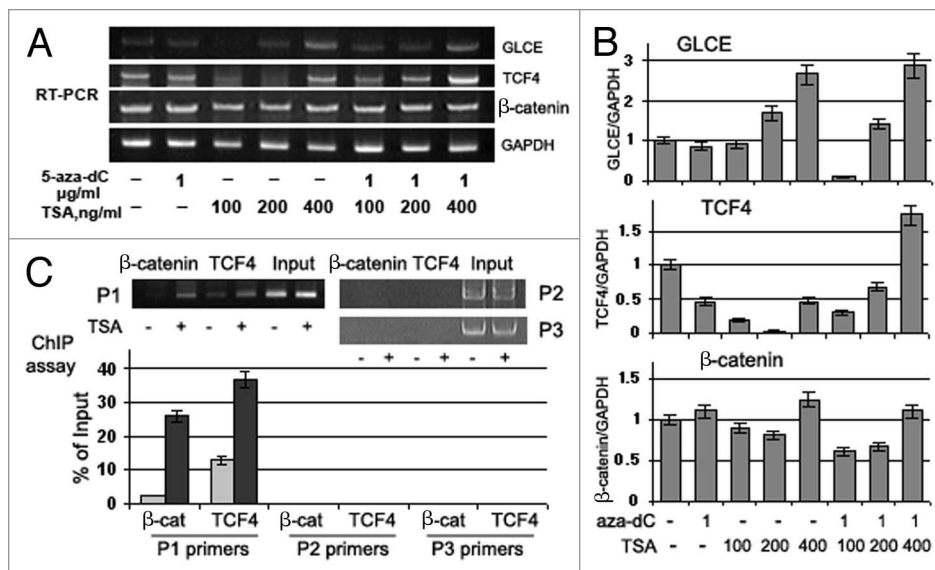


Figure 7. Effects of Trichostatin A on *GLCE*, *TCF4* and β -catenin expression and *GLCE* transcriptional activation in MCF7 breast carcinoma cells in vitro. (A) *GLCE*, *TCF4* and β -catenin expression upon treatment with 5-aza-deoxycytidine (5-aza-dC) and/or Trichostatin A (TSA). Representative multiplex RT-PCR electropherogram with *GAPDH* gene as an internal control. (B) Intensity of the amplified DNA fragments normalized to that of *GAPDH*. Bars represent the mean \pm SD of triplicate experiments (Orig-Info 8.1). (C) ChIP assay for the *GLCE* promoter region with anti-TCF4 or anti- β -catenin antibodies. DNA fragments corresponding to TCF4-responsive region of the *GLCE* promoter were amplified using P1 primers, TCF4-non-responsive promoter regions were amplified with P2 and P3 primers as control. The amount of immunoprecipitated DNA was normalized to that of the input DNA, TSA concentration was 400 ng/ml.

and *GAPDH* (housekeeping gene) were amplified for 32 and 20 cycles, respectively. The PCR primers used were: *GLCE*-F, 5'-AAG GGA GAC GAG AGG GGA ACG AA-3'; *GLCE*-R, 5'-GCC ACC TTT CTC ATC CTG GTT C-3'; *GAPDH*-F, 5'-GGG CGC CTG GTC ACA A-3'; *GAPDH*-R, 5'-AAC ATG GGG GCA TCA GCA GA-3'. The following conditions were used for multiplex RT-PCR for *TCF4* and *CTNNB1*: 95°C for 10 min, 95°C for 15 sec, 59°C for *TCF4* or 61°C for *CTNNB1* for 20 sec, 72°C for 1 min, and 72°C for 10 min using a Tercik PCR machine (DNA-technology, Russia). The total reaction volume was 20 μl . *TCF4*, *CTNNB1* and *GAPDH* were amplified for 32, 29 and 20 cycles, respectively. The PCR primers were: *TCF4*-F: 5'-CCA GTG AAG GTG TAT CCT TGC AAC T-3'; *TCF4*-R: 5'-GCC ACC TTT CTC ATC CTG GTT CC-3'; *CTNNB1*-F: 5'-CCA GTG AAG GTG TAT CCT TGC AAC T-3'; *CTNNB1*-R: 5'-CTA CCG GTG TGC TTT GCC CTG CTG C-3'. The amplified products were separated on 1.2% agarose gels. The gels were scanned using the “DNA Analyzer” system (Vilber Lourmat) and *GLCE*, *TCF4* or *CTNNB1* expression levels were estimated from the intensity of the amplified DNA fragments normalized against the intensity of *GAPDH* using the TotalLab program (Nonlinear Dynamics).

Analysis of *GLCE* expression by quantitative TaqMan-based Real-Time RT-PCR. Quantitative real-time RT-PCR (qRT-PCR) was performed using the BioRad IQ5 Multicolor Real-Time PCR Detection System (BioRad) and the *GLCE* TaqMan Custom Assay (Applied Biosystems) under the following

conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec. The total reaction volume was 25 μ l. β -actin (*ACTB*) was used as the housekeeping gene. The PCR primers and TaqMan probes used were: GLCE-F, 5'-TTC CAA AGT CTA TGC ACA GAG AGC-3'; GLCE-R, 5'-TCC ACA TTG TAG CCT TCA AAA GAC A-3'; GLCE-probe, 5'-FAM-CCC CTA TCA CCC CGA TGG T-TAMRA-3'; β -actin-F, 5'-GGC ACC CAG CAC AAT GAA G-3', β -actin-R, 5'-GCC GAT CCA CAC GGA GTA CT-3', β -actin-probe, 5'-FAM-TCA AGA TCA TTG CTC CTC CTG AGC GC-TAMRA-3'.

Genomic DNA isolation and bisulphite conversion. Genomic DNA was isolated from the tissue samples using the E.Z.N.A. DNA isolation kit and bisulphite conversion of the genomic DNA was performed using an E.Z.N.A. DNA methylation kit (Zymo Research) according to the manufacturer's instructions.

Methyl-specific PCR. Methyl-specific PCR for GLCE fragment amplification was performed with primers specific for the methylated (M) and unmethylated (U) DNA sequences within *GLCE* CpG islands. Blood genomic (g)DNA, treated with SssI methyltransferase (New England Biolabs), was used as a positive control for the Met-primers. The following conditions were used for PCR: 95°C for 4 min, followed by 45 cycles of 95°C for 30 sec, 60–65°C for 30 sec, and 72°C for 30 sec, with a final elongation step at 72°C for 7 min. The reaction mixture contained 1 \times DreamTaq buffer, 0.2 mM dNTP's, 0.4 μ M primers, 3.5% DMSO, 50–100 ng of bisulphite-converted DNA and 1.25 U of DreamTaq DNA-polymerase (Fermentas). The total reaction volume was 30 μ l; 10 μ l of the amplified products were separated on 10% polyacrylamide (PAGE) and visualized by ethidium bromide staining. The PCR primers were: GLCE-M-F, 5'-TTG GTC GTA GTA GAT TTC GAG TTT TGT C-3'; GLCE-M-R, 5'-CGC GCA ACC GAA AAA CCG-3'; GLCE-U-F, 5'-TTG AGT TTT GTT GTT TGT TTT GTA GTT-3'; GLCE-U-R, 5'-TAT AAA AAA AAC CCT CCC ACT CCA-3'.

Bisulphite sequencing. Amplification of the *GLCE* DNA fragment for bisulphite sequencing was performed using bisulphite-treated gDNA and primers specific for two overlapping *GLCE* CpG-island sequences, BS1 and BS2 (GLCE-BS1-F, 5'-GTA TTT TAA TAA TGG TGT TTT GTT TGA G-3'; GLCE-BS1-R, 5'-CCA AAA ATA ATA AAA AAC AAT AAA CTT TC-3'; GLCE-BS2-F, 5'-GAA AGT TTA TTG TTT TTT ATT ATT TTT GGT-3'; GLCE-BS2-R, 5'-ACC CCC AAA ATC CCT AAT ACA TTA C-3'). The following conditions were used for PCR: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min, with a final elongation step at 72°C for 10 min. The total reaction volume was 30 μ l. The PCR products were purified using a DNA Clean and Concentrator Kit (Zymo Research) and cloned into a TOPO-vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated using a Zyppy Plasmid Miniprep Kit (Zymo Research) according to the manufacturer's protocol. Sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 and ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol (8–10 clones were analyzed for each sample).

Western blotting. Cells were lysed with RIPA-buffer (1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris, pH 7.4) containing "Complete" Protease Inhibitor Cocktail (Roche), sonicated and centrifuged for 10 min at 12,000 g. The protein concentration was quantified using Quant-iT Protein Assay Kit (Invitrogen). Total proteins (30 μ g) were treated with NuPAGE LDS Sample Buffer (Invitrogen) containing 10% β -mercaptoethanol for 5 min at 100°C, resolved in 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h and incubated with primary antibodies [rabbit anti-GLCE polyclonal serum (GenScript Corporation), 1:2,000; mouse anti-H3K4Me3 (Abcam), 1:500; mouse anti-H3K9Me3 (Abcam), 1:500 or rabbit anti-H3K9ac (Invitrogen), 1:500] overnight at 4°C followed by secondary peroxidase-conjugated antibodies [goat anti-Mouse IgG (Abcam) or polyclonal goat anti-rabbit (Pierce)] for 1 h at RT. Proteins were detected with an Opti-4CN Substrate Kit (Bio-Rad) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay. ChIP was performed using a MAGnify Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer's instructions. Briefly, cells were rinsed in cold PBS and treated with 1% formaldehyde at RT for 10 min and lysed in lysis buffer containing protease inhibitors for 5 min on ice. Lysates were sonicated on ice in a Microson™ Ultrasonic Liquid Processor XL-2,000 cell disrupter, yielding chromatin fragments of 500–1,000 bp. Samples were centrifuged at 13,000 \times g for 10 min and the supernatants were collected. To provide a positive control (Input), 20 μ l of the supernatants were retained. The supernatants were diluted 10-fold with dilution buffer. Protein A/G mix Dynabeads were incubated with 3 μ g of monoclonal antibodies against modified histones, H3K9ac, H3K9me3, H3K4me3, and TCF4 and β -catenin, or with normal rabbit or mouse IgG for 1 h at 4°C followed by precipitation with a DynalMPC-S magnet (Invitrogen). Precipitates were incubated with 100 μ l of diluted cell lysate for 2 h at 4°C. The Dynabeads were then washed with IP1 and IP2 washing buffers and incubated with Stock Reverse Crosslinking buffer containing proteinase K for 15 min at 55°C. The supernatant was transferred into fresh Eppendorf tubes and incubated for 15 min at 95°C. PCR amplification was performed using three primer pairs specific for the *GLCE* promoter site: TCF4-binding region-specific primers P1-F 5'-TCC CAA GAA GTG TGA TTC CTA GGA GA-3', P1-R 5'-TCA CCC ACA GCA AGA AGT GCC CT-3'; P2-F 5'-GCC ACC CCT TCT CTG CTG TC-3', P2-R 5'-GCT CCG GGC TTT CTA ACC TG-3' and P3-F 5'-GCA CTT CTT GCT GTG GGT GAG-3', P3-R 5'-TTC CTA GCC TTT CCT CTA TAA TGT GAG-3'. The PCR products were analyzed by PAGE electrophoresis. The gels were scanned using the "DNA Analyzer" system (Vilber Lourmat) and the interaction between histones and the *GLCE* promoter sequence was estimated from the intensity of the amplified *GLCE* fragment normalized against the intensity of the input control (TotalLab program, Nonlinear Dynamics).

Wnt signaling pathway RT² profiler PCR array. The WNT Signaling Pathway PCR Array (SABioscience) was used

to determine changes in the expression of 84 Wnt pathway-focused genes upon TSA treatment in MCF7 cells. Briefly, total RNA was isolated using an RNAqueous Micro Kit (Applied Biosystems). The RNA concentration was determined using a Quant-iT Assay Kit (Invitrogen) and was verified by electrophoresis. cDNA was synthesized from 1–2 µg of total RNA using a First Strand cDNA Synthesis kit (Fermentas). Real-Time PCR was performed using a WNT Signaling Pathway PCR Array with SYBR Green PCR Master Mix (Fermentas) and an iCycler iQ5 Multicolor Detection System (Bio-Rad) according to the manufacturer's instructions. All data were analyzed using Excel-based PCR Array Data Analysis Software (SABioscience). This integrated web-based software package automatically calculates the $\Delta\Delta C_t$ -based fold changes in gene expression from the uploaded raw threshold cycle data. Each replicate cycle threshold (C_t) was normalized to the average C_t of five endogenous controls (B2M, HPRT1, RPL13A, GAPDH and ACTB) on a per plate basis. The obtained data were analyzed using a companion scoring algorithm that converts gene expression data into quantitative measurements of pathway activity.

Immunocytochemistry. For immunofluorescence analysis, cells were grown on glass coverslips and then transfected with human TCF4 and/or β -catenin pcDNA3-expressing plasmids and the appropriate control plasmids using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's instructions. pcDNA/Myc TCF4 and pcDNA/Myc DeltaN TCF4 were kindly provided by Dr B. Vogelstein (Johns Hopkins University and the Howard Hughes Medical Institute), human β -catenin pcDNA3 was kindly provided by Dr E.R. Fearon (Regents of the University of Michigan), pcDNA3-EGFP was kindly provided by Dr D. Golenbock (University of Massachusetts,

Worcester). After 36 h, the transfected cells were fixed with phosphate-buffered 4% formaldehyde. Anti-GLCE custom rabbit polyclonal serum (GenScript Corporation; 1:100), mouse monoclonal anti-TCF4 (Abcam; 1:100) and rabbit monoclonal anti- β -catenin (Abcam; 1:500) were used for immunostaining. Staining patterns were visualized with Alexa 488-conjugated goat anti-mouse IgG and Alexa 546-conjugated goat anti-rabbit IgG (Invitrogen; 1:1000) antibodies. The cells were mounted and counterstained with DAPI using SlowFade Gold and DAPI mounting medium (Invitrogen) and observed under a fluorescence microscope (Axio Imager, Carl Zeiss).

Statistical analysis. Pearson's correlation was used to determine the association between *GLCE*, *TCF4* and β -*catenin* expression. Correlation coefficients (r) were calculated using a computer program ORIGIN Pro 8.0, $p < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr Bystrov A.A. and Grigoriev A.D. for the help with statistical analysis. The work was supported by the research grants from Russian Foundation for Basic Research (11–04–90400-Ukr_f_a); Ukrainian State Foundation of Fundamental Research (F40/146–2011, F46/457–2011); Swedish Institute (2011/00888); UICC International Cancer Technology Transfer Fellowship (TYP, ICRET-09–069); FEBS Short-term Fellowship (TYP); Karolinska Institute; ERZ was supported by the Swedish Cancer Society and Swedish Research Council.

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